Assay method of formalin

This is a method to determine the content of formalin in the sample from the absorbance at a wavelength of 410 nm utilizing that formaldehyde in formalin reacts acetylacetone and ammonia under slight acidic conditions, produces 3,5-diacetyl 1,4-dihydrolutidine and develops an orange-yellow color.

1. Standard solution and test solution

1.1. Standard formalin solution

Dilute formaldehyde solution exactly 500-fold with water.

1.2. Acetic acid-ammonium acetate buffer solution (pH 6.25)

1.2.1. Acetic acid solution

To 12.9 mL of acetic acid, add water to make 100 mL.

1.2.2. Ammonium acetate solution

Dissolve 173.4g of ammonium acetate in water to make 1,000 mL.

Mix 40 mL of the acetic acid solution and 1,000 mL of the ammonium acetate solution and store in a cold and dark place.

1.3. Acetylacetone test solution

Mix 7 mL of acetylacetone and 14 mL of dehydrated ethanol and add water to make 1,000 mL.

2. Test method

Dilute the test sample exactly with water so as to contain formalin in 0.01 to 0.05% and use this solution as the sample. Pipet 0.5, 1, 1.5, 2 and 2.5 mL of the standard formalin solution and add water to make exactly 10 mL. Use these solutions as the standard dilutions of 0.01, 0.02, 0.03, 0.04 and 0.05 vol%.

Pipet 0.1 mL each of the sample and each standard dilution, add 2 mL each of the acetic acid-ammonium acetate buffer solution, mix with 2 mL each of the acetylacetone test solution, and warm at 60℃ for 15 minutes. Cool these solutions with cold water for 5 minutes and allow to stand for 20 minutes. However, when the solution is turbid, centrifuge this solution at 1400×g or higher for 10 minutes. Determine the absorbance at a wavelength of 410 nm.

Prepare the calibration curve from the absorbance of the standard dilutions and extrapolate the absorbance of the sample to determine the content of formalin in the test sample. Separately, perform a blank determination using water as the control in a similar manner to determine the absorbance and make any necessary correction.

Moisture content test

This is a method to determine the water content in the test sample from the weight which is lost by heating and drying the dried product under reduced pressure.

1. Test method

Operate the test sample in an air at the relative humidity of 45% or less. Weigh accurately a weighing bottle that has previously been dried. Weigh accurately about 100 mg of the ground test sample in a weighing bottle and use as the sample.

Place the weighing bottle into a vacuum drying chamber as the surface of the mouth is slightly opened and dry the test sample at 60℃ for 3 hours under reduced pressure of 0.65 kPa or lower on phosphorus pentoxide or silica gel if necessary.

At completion of drying, place the dried air into a vacuum drying chamber to return to the atmospheric pressure, transfer the weighing bottle into a desiccator containing phosphorus pentoxide or silica gel, cool to ordinary temperature, and weigh accurately the weighing bottle.

Calculation of moisture content

Calculate the moisture content according to the following equation:

$$\text{Moisture content (\%)} = \frac{\text{Weight lost on drying}}{\text{Original weight of the sample}} \times 100$$

2. Judgment

As a result of the test, unless otherwise specified, consider to meet this test when the moisture content is 3% or less.

Sterility test method

Unless otherwise specified, this is a method to examine that there is no detectable microorganism in the test sample.

1. Bacteria negation test

1.1. Medium

Unless otherwise specified, use the thioglycollate broth of the following composition. The volume of the medium shall be usually 15 mL or more per tube.

1.1.1. Thioglycollate broth

1.1.1.1. Composition

In 1,000 mL:

L-cystine	0.5g
Agar	0.8g
Sodium chloride	2.5g
Glucose	5.0g
Yeast extract	5.0g
Casein-peptone	15.0g
Sodium thioglycollate	0.5g
0.1 W/V% Resazurin solution	1.0 mL
Water	q.s.

Autoclave the broth at 121℃ for 15 minutes, cool rapidly, and store in a dark place of 25℃. Adjust the pH after sterilization to 7.0 to 7.2.

The dry product of appropriate quality may be used.

1.1.1.2. Performance

Perform the performance test of the medium according to the following tests:

1.1.1.2.1. Growth test

Inoculate less than 100 bacteria each of hemolytic streptpcocci and Clostridium sporogenes and incubate at 30 to 32℃ for 72 hours: The bacteria should proliferate sufficiently.

1.1.1.2.2. Oxidation-reduction test

When the red-pink layer of the medium is 30% or less in the upper part and allowed to stand at 30 to 32℃ for 7 days, 60% or more of the upper part of the medium should not be discolored.

1.2. Materials for culture

Use the test sample and the test material. And for the lyophilized product with no attached solvent, dissolve the sample with appropriate solvent such as phosphate

buffer to the specified amount described in Dosage and Administration.

1.3. Number of the test samples

Unless otherwise specified, take sufficient amount of the test sample from all the containers when the test sample is tested.

Unless otherwise specified, perform the test on 7 or more containers when the test material is tested.

1.4. Amount to be inoculated onto the medium

In case of the test sample, use 4 tubes containing the medium for each material and inoculate 1 mL each of the material onto 2 tubes and 0.5 mL each onto the other 2 tubes.

In the test on the test material, the labeled volume of the dispensing container and the amount to be inoculated onto the medium shall be as shown in Table 1.

Table 1. The labeled volume of the dispensing container and the number of inoculations per container (bacteria negation test)

1.5. Incubation and observation

Inoculate the test sample onto the medium, mix thoroughly, incubate at 30 to 32℃ for 14 days or more, and observe the presence or absence of bacterial growth on 3, 7 and 14 days during the period.

However, in the case when the medium became turbid due to preparation and in the case required, subculture onto a new medium on 7 days, incubate at the same temperature for 8 days or more, and observe.

1.6. Judgment

Consider the sample to meet this test when no bacterial growth is observed as a result of the test.

2. Fungi negation test

2.1. Medium

Unless otherwise specified, use the thioglycollate broth. For the test sample not containing timerosal, unless otherwise specified, use the soybean casein digest broth.

The volume of the medium shall be usually 15 mL or more per tube.

2.1.1. Thioglycollate broth

2.1.1.1. Composition

Apply the medium described in 1.1.1.1.

2.1.1.2. Performance

Perform the performance test of the medium according to the following tests:

2.1.1.2.1. Growth test

Inoculate less than 100 bacteria of hemolytic streptococci and less than 100 each

conidia of Aspergillus niger and Candida albicans and incubate at 20 to 25℃ for 5 days: The bacteria should proliferate sufficiently.

2.1.1.2.2. Oxidation-reduction test

Apply the test described in 1.1.1.2.2.

2.1.2. Soybean casein digest broth

2.1.2.1. Composition

In 1,000 mL:

Casein peptone	17.0g
Soybean peptone	3.0g
Sodium chloride	5.0g
Dibasic potassium phosphate	2.5g
Glucose	2.5g
Water	q.s.

Autoclave the broth at 121℃ for 15 minutes and adjust the pH after sterilization to 7.2 to 7.4. And the dry product of appropriate quality may be used.

2.1.2.2. Performance

Apply the test described in 2.1.1.2.1.

2.2. Materials for culture

Apply the materials for culture described in 1.2.

2.3. Number of the test samples

Apply the number of the test samples described in 1.3.

2.4. Amount to be inoculated onto the medium

In case of the test sample, use 4 tubes containing the medium for each material and inoculate 1 mL each of the material onto the tubes.

In the test on the test material, the labeled volume of the dispensing container and the amount to be inoculated onto the medium shall be as shown in Table 2.

Table 2. The labeled volume of the dispensing container and the number of inoculations per container (fungi negation test)

2.5. Incubation and observation

Inoculate the test sample onto the medium, mix thoroughly, incubate at 20 to 25℃ for 14 days or more, and observe the presence or absence of fungal growth on 3, 7 and 14 days during the period.

However, in the case when the medium became turbid due to preparation and in the case required, subculture onto a new medium on 7 days, incubate at the same temperature for 8 days or more, and observe.

2.6. Judgment

Apply the judgment described in 1.6.

3. Detection of mycoplasma contamination

3.1. Direct culture method

3.1.1. Medium

Unless otherwise specified, use the mycoplasma broth of the following composition. The volume of the medium shall be usually 100 mL per bottle.

3.1.1.1. Mycoplasma growth medium

3.1.1.1.1. Composition

3.1.1.1.1.1. Basal medium

In 1,000 mL:

50 W/V% Bovine myocardial effusion	100 mL
Beef peptone	10g
Sodium chloride	5g
Glucose	1g
Sodium L-glutamate	0.1g
L-Arginine hydrochloride	1g
Water	q.s.

Sterilize the broth by filtering through a membrane filter of 0.22 μ m, or autoclave at 121℃ for 15 minutes. Adjust the pH after sterilization to 7.2 to 7.4.

And the dry product of appropriate quality may be used.

3.1.1.1.1.2. Additives to medium

To 77 mL of the basal medium described in 3.1.1.1.1.1, add the following components:

Equine serum	10 mL
Inactivated porcine serum	5 mL
25 W/V% Fresh yeast extract	5 mL
1 W/V% β -nicotinamide adenine dinucleotide [oxidized type] solution	1 mL
1 W/V% L-cystine hydrochloride [monohydrate] solution	1 mL
0.2 W/V% Phenol red solution	1 mL

To the sterilized basal medium, add aseptically each of the above additives previously sterilized by filtration.

And the additive which can be sterilized by an autoclave may be autoclaved.

And penicillin G potassium and potassium acetate may be added at 500 units in 1 mL

of the medium and at 0.02 W/V%, respectively.

3.1.1.1.2. Performance

Inoculate less than 100 CFU each of Mycoplasma gallisepticum, M. synoviae, M. hyopneumoniae and M. orale and incubate at 35 to 37℃ for 7 days: The mycoplasma colonies should grow sufficiently .

3.1.1.2. Mycoplasma agar

3.1.1.2.1. Composition

3.1.1.2.1.1. Basal medium

To 78 mL of the basal culture medium described in 3.1.1.1.1.1, add 1g of agar.

3.1.1.2.1.2. Additives to medium

Exclude the phenol red solution from the additives to medium described in 3.1.1.1.1.2.

After sterilization, dispense 5 mL each of the medium dissolved by heating into the sterilized petri dishes of 45 to 55 mm in diameter, cool, allow to coagulate, and use as the Mycoplasma agar plate.

3.1.1.2.2. Performance

Applying the performance described in 3.1.1.1.2, inoculate less than 100 CFU each of Mycoplasma strains, incubate at 35 to 37℃ for 10 days under the atmosphere of 5 vol% carbon dioxide gas: The strains should form inherent colonies.

3.1.2. Preparation of inoculum

Apply the materials for culture described in 1.2. And in the case of the drugs to be administered by drinking water and a puncture route, dilute the vaccine with appropriate diluent such as phosphate buffer solution so as to make one dose per inoculation.

3.1.3. Number of the test samples

Collect and mix an equivalent amount of the test sample from each container or of the test material from 2 or more dispensed containers.

3.1.4. Amount to be inoculated onto the medium

Inoculate 1 mL of the test sample onto 100 mL of broth. And from broth to agar plate, inoculate 0.1 mL each.

3.1.5. Incubation and observation

Inoculate the test sample onto the broth, mix thoroughly, and incubate at 35 to 37℃ for 14 days. And in the case of the test sample containing vital cells, adjust the pH of the broth as appropriate.

Inoculate the broth onto the mycoplasma agar plate on 3, 7, 10 and 14 days after incubation, incubate at 35 to 37℃ for 10 days under the atmosphere of 5 vol% carbon dioxide gas, and observe the presence or absence of the colony of mycoplasma. In this

case, however, observe simultaneously the plate onto which the medium and M. synoviae were inoculated as the control.

3.1.6. Judgment

Consider the sample to meet this test when no colony of mycoplasma was observed in the plate onto which the test sample and the medium were inoculated and when colony formation was observed in the plate onto which M. synoviae was inoculated as a result of the test.

Repeat the test when no colony was observed in the plate onto which M. synoviae was inoculated and when colony formation was observed in the plate onto which the medium was inoculated.

3.2. Gene amplification method(PCR method)

3.2.1. Medium

Unless otherwise specified, use the mycoplasma broth of the following composition. The volume of the medium shall be usually 100 mL per tube.

3.2.1.1. Mycoplasma growth medium

Apply the broth described in 3.1.1.1.

3.2.1.2. Preparation of inoculum

Apply the materials for culture described in 3.1.2.

3.2.1.3. Number of the test samples

Collect and mix an equivalent amount of the test sample from each container or of the test material from 2 or more dispensed containers.

3.2.1.4. Amount to be inoculated onto the medium

Inoculate 1 mL of the test sample onto 100 mL of broth.

3.2.1.5. Incubation

Inoculate the test sample onto the broth, mix thoroughly, and incubate at 35 to 37℃ for 7 days. And in the case of the test sample containing vital cells, adjust the pH of the broth as appropriate.

In this case, however, incubate simultaneously the plate onto which the medium and M. synoviae were inoculated as the control.

3.2.1.6. PCR method

3.2.1.6.1. DNA extraction method

3.2.1.6.1.1. DNA extract

50 mM	Potassium chloride
2.5 mM	Magnesium chloride
0.01 W/V%	Gelatin
0.45 vol%	Noidet-P40

0.45 vol%	Polysorbate 20
4 units/mL	Proteinase K
10 mM	Tris-hydrochloride buffer

Adjust the pH of the extract to 8.3, autoclave the components except for proteinase K at 121℃ for 15 minutes, and mix aseptically. Store the extract at -20℃.

3.2.1.6.1.2. Extraction of DNA

Centrifuge 0.5 mL of the medium described in 3.2.1.5 at 15,000×g and 4℃ for 20 minutes, remove the supernatant, and add 40 μl of the DNA extract. React this mixture at 60℃ for 20 minutes and then at 94℃ for 10 minutes.

3.2.1.6.2. PCR

3.2.1.6.2.1. PCR solution

Sterilized distilled water	34.75 μl
10-fold concentrated PCR buffer solution (Appendix 1)	5 μl
Dinucleotide triphosphate mixture	4 μl

(mixture of an equivalent amount of 2.5 mM each dAPT, dCTP, dGTP and dTTP)

10 pmol/μl Primer MP1	0.5 μl
10 pmol/μl Primer MP2	0.5 μl
5 units/μl Taq DNA polymerase	0.25 μl

However, the sequence of primer is as follows:

MP1: 5'-GCTGCGGTGAATACGTTCT-3'

MP2: 5'-TCCCCACGTTCTCGTAGGG-3'

3.2.1.6.2.2. Gene amplification

Add 5 μl of the DNA extract to 45 μl of the PCR solution, achieve the amplification with 30 cycles of denaturation at 94℃ for 30 sec, annealing at 50℃ for 30 sec and extension at 72℃ for 5 min, and follow by a final extension at 72℃ for 1 min.

3.2.1.6.3. Agarose gel electrophoresis

3.2.1.6.3.1. Buffer solution for electrophoresis

In 1,000 mL:

Tris[hydroxymethyl]aminomethane	10.8g
Boric acid	5.5g
0.5N disodium ethylenediamine tetraacetate (pH 8.0)	5 mL
Water	q.s.

3.2.1.3.2. Electrophoresis

Dissolve agarose of appropriate quality in the buffer solution for electrophoresis by heating so as to be 2 W/V% and allow to coagulate in the appropriate submarine type electrophoretic chamber.

Mix 8 μ l of the reactant solution after gene amplification with 2 μ l of the dye solution (Appendix 2) and inject the whole amount into the sample groove. And simultaneously prepare an appropriate DNA molecular weight marker by which the molecular weight of DNA of about 160 base pairs can be identified. Perform electrophoresis at a constant voltage of 100V, stain with the ethidium bromide solution (Appendix 3) for 15 minutes, and identify the DNA band by UV irradiation.

3.2.1.6.4. Judgment

Consider the test sample to meet this test when no DNA band corresponding to about 160 base pairs of the genetic sequence specific for mycoplasma was observed in the medium onto which the test sample and the medium were inoculated and when specific DNA band was observed in the medium onto which M. synoviae was inoculated as a result of the test.

Repeat the test when no specific DNA band was observed in the medium onto which M. synoviae was inoculated and when DNA band was observed in the medium onto which only the medium was inoculated.

4. Salmonella negation test

4.1. Medium

Unless otherwise specified, use soybean casein digest broth (SCD broth), selenite medium, BTB lactose agar (modified Drigalski medium) and DHL agar.

The volume of the broth shall be usually 100 mL per tube.

4.1.1. Soybean casein digest broth

4.1.1.1. Composition

Dissolve the dry product of appropriate quality according to the description and autoclave at 121℃ for 15 minutes. Adjust the pH after sterilization to 7.1 to 7.3.

4.1.1.2. Performance

Inoculate less than 100 each bacteria of Escherichia coli and Salmonella typhimurium and incubate at 35 to 37℃ for 18 to 24 hours: The bacteria should proliferate sufficiently.

4.1.2. Selenite medium

4.1.2.1. Composition

Dissolve the dry product of appropriate quality according to the description. Adjust the pH to 7.1 to 7.3.

4.1.2.2. Performance

Inoculate less than 100 bacteria of S. typhimurium and incubate at 35 to 37℃ for 18 to 24 hours: The bacteria should proliferate sufficiently.

4.1.3. BTB lactose agar

4.1.3.1. Composition

Dissolve the dry product of appropriate quality according to the description and autoclave at 121℃ for 15 minutes. Adjust the pH to 7.3 to 7.5.

4.1.3.2. Performance

Inoculate less than 100 each bacteria of E. coli, S. pullorum and S. typhimurium and incubate at 35 to 37℃ for 18 to 24 hours: Respective inherent colonies should be formed.

4.1.4. DHL agar

4.1.4.1. Composition

Dissolve the dry product of appropriate quality according to the description. Adjust the pH to 6.9 to 7.1.

4.1.4.2. Performance

Inoculate less than 100 each bacteria of E. coli, S. pullorum and S. typhimurium and incubate at 35 to 37℃ for 18 to 24 hours: Respective inherent colonies should be formed.

4.2. Materials for culture

Apply the materials for culture described in 3.1.2.

4.3. Number of the test samples

Apply the number of the test samples described in 3.3.

4.4. Incubation and observation

Inoculate 5 mL each of the test sample to the SCD broth and the selenite medium, mix thoroughly and perform enrichment incubation at 35 to 37℃ for 18 to 24 hours. Inoculate 0.1 mL each of the media onto the BTB lactose agar plate and the DHL agar plate, incubate at 35 to 37℃ for 18 to 24 hours, and examine the presence or absence of the colony of Salmonella.

4.5. Judgment

Consider the test sample to meet this test when no colony of Salmonella is observed as a result of the test.

5. Retest

When the results of the tests 1, 2, 3 and 4 are doubtful, the tests should be repeated newly using more than the twice amount of the test sample.

Appendix 1. 10-fold concentrated PCR buffer solution

100 mM	Tris-hydrochloride buffer solution (pH 8.3)

500 mM	Potassium chloride
15 mM	Magnesium chloride

Appendix 2. Dye solution

Phycol (Type 400)	2.5g
Bromphenol blue	0.025g
Buffer solution for electrophoresis	10 mL

After dissolution, store at 4℃, and mix thoroughly by stirring before use.

Appendix 3. Ethidium bromide solution

Dissolve ethidium bromide in distilled water to the concentration of 5 mg/ml and store at 4℃ in a light resistant container. Dilute to 10,000-fold before use.

Detetion of extraneous viruses in the biological products

This is a method to investigate that no aberrant virus which can be detected in the samples such as live vaccine and sera is present.

1. Sample

1.1. Live vaccine

Unless specified in the monograph, to the test sample and the liquid test sample as they are or to the dried test sample which was dissolved in the attached solution for dissolution or a phosphate buffered saline solution, add each immune serum shown in the monograph, incubate at 37℃ for 1 hour or at 4℃ overnight, and use the test sample which was completely neutralized as the sample.

Unless specified in the monograph, adjust the amount of virus for a single inoculation so that the virus for 1 animal (chicken) is contained as the amount of virus before neutralization.

1.2. Sera

Dialyze the test sample with 100-fold or more amount of a phosphate buffered saline solution at 2 to 5℃ overnight, and use the test sample from which a preservative was removed as the sample.

2. Test method

2.1. Growing egg inoculation test

2.1.1. Intraallantoic inoculation test

2.1.1.1. Observation of chicken embryo

2.1.1.1.1. Test method

Use 9- to 11-day old growing eggs prescribed in 1.1 of the specifications of the materials for manufacturing of live vaccine. Inject 0.1 mL each of the sample into the allantoic cavity of 10 growing eggs and incubate at 37℃ for 7 days. Open the egg on the last day of incubation and observe the presence or not of the abnormality of chicken embryo.

2.1.1.1.2. Judgment

Consider the sample to meet this test when the chicken embryo grow normally and show no abnormality.

2.1.1.2. Hemagglutination test

2.1.1.2.1. Test method

To the allantoic fluid collected on the last day of incubation described in 2.1.1.1, add an equivalent amount of 0.5 vol% chicken red blood cell suspension, allow to stand at 4℃ for 60 minutes, and observe the presence or not of hemagglutination.

2.1.1.2.2. Judgment

Consider the sample to meet this test when no hemagglutination is observed in the

allantoic fluid.

2.1.2. Chorioallantoic membran inoculation test

2.1.2.1. Test method

Use 10- to 12-day old growing eggs prescribed in 1.1 of the specifications of the materials for manufacturing of live vaccine. Inject 0.1 mL each of the sample onto the chorioallantoic membran of 10 growing eggs and incubate at 37℃ for 5 days. Open the egg on the last day of incubation and observe the presence or not of the abnormality of chicken embryo and chorioallantoic membran.

2.1.2.2. Judgment

Consider the sample to meet this test when the chicken embryo grow normally and the chorioallantoic membran show no abnormality.

2.2. Chicken-derived cell inoculation test

2.2.1. Cultured chicken kidney cell inoculation test

2.2.1.1. Observation of cells

2.2.1.1.1. Test method

Use the primary chicken kidney cells prescribed in 2.2.1 of the specifications of the materials for manufacturing of live vaccine. Culture the cells in 4 or more petri dishes of 20 cm^2 or more, inoculate 0.2 mL each of the sample, and incubate at 37℃ for 7 days. Collect the culture medium and the cells, inoculate and subculture 0.2 mL each of the medium on 4 or more petri dishes for the cultured cells, incubate at 37℃ for 7 days, and observe the presence or not of CPE.

2.2.1.1.2. Judgment

Consider the sample to meet this test when no CPE is observed in the cultured cells during the observation period.

2.2.1.2. Hemadsorption test

2.2.1.2.1. Test method

On the last day of observation in 2.2.1.1, remove the medium from the petri dish for cultured cell, stratify 0.1 vol% chicken red blood cell suspension, allow to stand at 4℃ for 60 minutes, and observe the presence or not of hemadsorption under microscopy.

2.2.1.2.2. Judgment

Consider the sample to meet this test when no hemadsorption is observed in the cultured cell.

2.2.2. Cultured chicken embryonic cell inoculation test

2.2.2.1. Observation of cells

2.2.2.1.1. Test method

Use the primary chicken embryonic cells prescribed in 2.2.1 of the specifications of the materials for manufacturing of live vaccine susceptible to chicken leukemia virus.

Culture the cells in 8 or more petri dishes of 20 cm^2 or more, inoculate 0.2 mL each of the sample within 24 hours, and incubate at 37℃. Subculture these cells every 3 to 5 days until the third passage, and observe the presence or not of CPE on the cells in each generation.

2.2.2.1.2. Judgment

Consider the sample to meet this test when no CPE is observed in the cultured cells during the observation period.

2.2.2.2. Hemadsorption test

2.2.2.2.1. Test method

For 4 or more petri dishes for the cultured cells in the third passage in 2.2.2.1, remove the medium, stratify 0.1 vol% chicken red blood cell suspension, allow to stand at 4℃ for 60 minutes, and observe the presence or not of hemadsorption.

2.2.2.2.2. Judgment

Consider the sample to meet this test when no hemadsorption is observed in the cultured cell.

2.2.2.3. COFAL test

2.2.2.3.1. Test method

Wash the cells in 4 petri dishes for the cultured cells in the third passage in 2.2.2.1, the cell is scraped with 1% gelatinized Veronal buffer solution in the 1/20 amount of the medium.The cell suspension is freezed and thawed at three times. After centrifuzation,the supernatant is collected as the cell exatract. Use this extract as the antigen and perform the COFAL test by Kolmer method using the sera of RSV cancer carrying pigeon, rabbit or hamster, 3 units of hemolysin, 4 units of guinea pig complement and 2 vol% sheep red blood cells.

2.2.2.3.2. Judgment

Consider the sample to meet this test when the cell extract is negative in the COFAL test.

2.2.2.4. Reticuloendotheliosis virus negation test

2.2.2.4.1. Test method

Inoculate the sample in 2.2.2.1.1, inoculate the cultured primary cell suspension to the separately and newly cultured primary chicken embryonic cells, and incubate at 37℃ for 4 days. In order to subculture the cell suspension to the third passage, inoculate onto 4 or more petri dishes with a coverslip for cultured cells, incubate at 37 ℃ for 4 days, perform the fluorescent antibody technique against reticuloendotheliosis virus, and observe by the UV excitation mode.

2.2.2.4.2. Judgment

Consider the sample to meet this test when no specific fluorescent antigen is

observed in the cultured cells.

2.3. Pig-derived cell inoculation test

2.3.1. Cultured porcine kidney cell inoculation test

2.3.1.1. Observation of cells

2.3.1.1.1. Test method

Use the primary or established porcine kidney cells.

Inoculate 2 mL of the sample to the cultured cells of 20 cm^2 or more per mL, incubate at 37℃ for 5 days, and observe the presence or not of CPE. Then, subculture the cells to the next passage, incubate at 37℃ for 7 days, and observe the presence or not of CPE.

2.3.1.1.2. Judgment

Consider the sample to meet this test when no CPE is observed in the cultured cells during the observation period.

2.3.1.2. Hemadsorption test

2.3.1.2.1. Test method

On the last day of observation in 2.3.1.1, wash the surface of the cultured cells twice with a phosphate buffered saline solution, divide the cultured cells into 3 groups, stratify 0.1 vol% red blood cell suspensions of guinea pig, goose and chicken within 7 days of age on each group, allow to stand at 4℃ for 60 minutes and at 37℃ for 30 minutes, and observe the presence or not of hemadsorption under microscopy.

2.3.1.2.2. Judgment

Consider the sample to meet this test when no hemadsorption is observed in the cultured cell.

2.3.1.3. Hog cholera virus negation test

2.3.1.3.1. Test method

Inoculate 2 mL of the sample to the cultured cells in 2.3.1.1, which were cultured on the coverslips of 3 cm^2 or more per 1 mL, incubate at 37℃ for 24 to 48 hours, perform the fluorescent antibody technique against hog cholera virus, and observe by the UV excitation mode.

2.3.1.3.2. Judgment

Consider the sample to meet this test when no specific fluorescent antigen is observed in the cultured cells.

2.3.2. Cultured porcine testicular cell inoculation test

2.3.2.1. Observation of cells

2.3.2.1.1. Test method

Use the primary or subcultured porcine testicular cells.

Inoculate 2 mL of the sample to the cultured cells of 20 cm^2 or more per mL, incubate

at 37℃, and observe for 10 days.

2.3.2.1.2. Judgment

Consider the sample to meet this test when no CPE is observed in the cultured cells during the observation period.

2.3.2.2. Hemadsorption test

2.3.2.2.1. Test method

On the last day of observation in 2.3.2.1, wash the surface of the cells twice with a phosphate buffered saline solution, divide the cultured cells into 3 groups, stratify 0.1 vol% red blood cell suspensions of guinea pig, goose and chicken within 7 days of age on each group, allow to stand at 4℃ for 60 minutes and at 37℃ for 30 minutes, and then observe the presence or not of hemadsorption under microscopy.

2.3.2.2.2. Judgment

Consider the sample to meet this test when no hemadsorption is observed in the cultured cell.

2.4. Bovine-derived cell inoculation test

2.4.1. Cultured bovine kidney cell inoculation test

2.4.1.1. Observation of cells

2.4.1.1.1. Test method

Use the subcultured bovine renal cells.

Inoculate 2 mL of the sample to the cultured cells of 20 cm^2 or more per mL, incubate at 34 to 36℃ for 5 days, and observe the presence or not of CPE. Then, subculture the cells to the next passage, wash the surface of the cells with a phosphate buffered saline solution when a monolayer was obtained, add the medium for maintenance, incubate at 37℃ for 5 days, and observe the presence or not of CPE.

2.4.1.1.2. Judgment

Consider the sample to meet this test when no CPE is observed in the cultured cells during the observation period.

2.4.1.2. Hemadsorption test

2.4.1.2.1. Test method

On the last day of observation in 2.4.1.1, divide the cultured cells into 2 groups, stratify 0.1 vol% red blood cell suspensions of guinea pig and goose, on each group, allow to stand for 60 minutes, and observe the presence or not of hemadsorption under microscopy.

2.4.1.2.2. Judgment

Consider the sample to meet this test when no hemadsorption is observed in the cultured cell.